Express Mail Label No. EL 358 311 628 US

UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No. 3045.00004
Total Pages in this Submission

TO THE ASSISTANT COMMISSIONER FOR PATENTS
Box Patent Application
Washington, D.C. 20231

Transmitted herewith for filing under 35 U.S.C. 111(a) and 37 C.F.R. 1.53(b) is a new utility patent application for an invention entitled:

ANTISENSE OLIGODEOXYNUCLEOTIDES REGULATING EXPRESSION OF TNF-ALPHA

and invented by:

Christopher Power and Michael B. Mayne

If a CONTINUATION APPLICATION, *check appropriate box and supply the requisite information:*

☒ **Continuation** ☐ **Divisional** ☐ **Continuation-in-part (CIP)** of prior application No.: **09/176,862**

Which is a:

☐ **Continuation** ☐ **Divisional** ☐ **Continuation-in-part (CIP)** of prior application No.: _____

Which is a:

☐ **Continuation** ☐ **Divisional** ☐ **Continuation-in-part (CIP)** of prior application No.: _____

Enclosed are:

Application Elements

1. ☒ Filing fee as calculated and transmitted as described below
2. ☒ Specification having **56** pages and including the following:
   - a. ☒ Descriptive Title of the Invention
   - b. ☒ Cross References to Related Applications *(if applicable)*
   - c. ☐ Statement Regarding Federally-sponsored Research/Development *(if applicable)*
   - d. ☐ Reference to Microfiche Appendix *(if applicable)*
   - e. ☒ Background of the Invention
   - f. ☒ Brief Summary of the Invention
   - g. ☒ Brief Description of the Drawings *(if drawings filed)*
   - h. ☒ Detailed Description
   - i. ☒ Claim(s) as Classified Below
   - j. ☒ Abstract of the Disclosure

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

"Express Mail" Mailing Label Number EL 358311628

Date of Deposit 11-15-99

I hereby certify that the papers or fees referred to are being deposited with the United States Postal Service Post Office to Addressee service under 37 CFR 1.10 on the date indicated above and are addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Constance McLean

(Signature of person mailing paper or fee)

UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No. 3045.00004
Total Pages in this Submission

Application Elements (Continued)

3. ☒ Drawing(s) *(when necessary as prescribed by 35 USC 113)*

   a. ☒ Formal b. ☐ Informal Number of Sheets 4

4. ☒ Oath or Declaration

   a. ☐ Newly executed *(original or copy)* ☐ Unexecuted

   b. ☒ Copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional application only)*

   c. ☒ With Power of Attorney ☐ Without Power of Attorney

   d. ☐ *DELETION OF INVENTOR(S)*
   Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. 1.63(d)(2) and 1.33(b).

5. ☒ Incorporation By Reference *(usable if Box 4b is checked)*
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. ☐ Computer Program in Microfiche

7. ☐ Genetic Sequence Submission *(if applicable, all must be included)*

   a. ☐ Paper Copy

   b. ☐ Computer Readable Copy

   c. ☐ Statement Verifying Identical Paper and Computer Readable Copy

Accompanying Application Parts

8. ☐ Assignment Papers *(cover sheet & documents)*

9. ☐ 37 CFR 3.73(b) Statement *(when there is an assignee)*

10. ☐ English Translation Document *(if applicable)*

11. ☐ Information Disclosure Statement/PTO-1449 ☐ Copies of IDS Citations

12. ☒ Preliminary Amendment

13. ☒ Acknowledgment postcard

14. ☒ Certificate of Mailing

    ☐ First Class ☒ Express Mail *(Specify Label No.):* EL 358 311 628 US

UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No. 3045.00004
Total Pages in this Submission

Accompanying Application Parts (Continued)

15. ☐ Certified Copy of Priority Document(s) *(if foreign priority is claimed)*

16. ☐ Small Entity Statement(s) - Specify Number of Statements Submitted: _____

17. ☒ Additional Enclosures *(please identify below):*

Copy of Small Entity Statements from parent case

Fee Calculation and Transmittal

CLAIMS AS FILED

For	#Filed	#Allowed	#Extra		Rate	Fee
Total Claims	14	- 20 =	0	x	$9.00	$0.00
Indep. Claims	5	- 3 =	2	x	$39.00	$78.00
Multiple Dependent Claims (check if applicable) ☐						$0.00
					BASIC FEE	$380.00
OTHER FEE *(specify purpose)* _____						$0.00
					TOTAL FILING FEE	$458.00

☒ A check in the amount of **$458.00** to cover the filing fee is enclosed.

☒ The Commissioner is hereby authorized to charge and credit Deposit Account No. **11-1449** as described below. A duplicate copy of this sheet is enclosed.

- ☐ Charge the amount of as filing fee.
- ☒ Credit any overpayment.
- ☒ Charge any additional filing fees required under 37 C.F.R. 1.16 and 1.17.
- ☐ Charge the issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance, pursuant to 37 C.F.R. 1.311(b).

Dated: **November 15, 1999**

Signature

Kenneth I. Kohn, Reg. No. 30,955
KOHN & ASSOCIATES
30500 Northwestern Highway, Suite 410
Farmington Hills, Michigan 48334
(248) 539-5050
FAX: (248) 539-5055

cc:

Attorney's Docket Number: 3045.00002 **PATENT**

Applicant or Patentee: Power et al.

Serial or Patent No.:

Filed or Issued: Herewith

Title: ANTISENSE OLIGODEOXYNUCLEOTIDES REGULATING EXPRESSION OF TNF-ALPHA

VERIFIED STATEMENT CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(d) — NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: The University of Manitoba

Organization Address: Administration Building - Room 202

Winnipeg, Manitoba, CANADA R3T 2N2

Type of Organization:

_X_ University or other Institution of Higher Education

____ Tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3)

____ Nonprofit Scientific or Educational under Statute of State of the United States of America State: _____
Citation of Statute: _____

____ Would Qualify as Tax Exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)3) if located int he United States of America (State: _____)
(Citation of Statute: _____)

I hereby declare that the nonprofit organization identified above qualified as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Section 41(a) and 41(b) of Title 35, of United States Code with regard to the invention as entitled above.

Described in:

_X_ the specification filed herewith with title as listed above.

____ the application identified above.

____ the patent identified above.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or nonprofit organization under 37 CFR 1.9(3).

- **Separate verified statements are required from each person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)**

Each such person, concern or organization having any rights int he invention is listed below:

____ No such person, concern, or organization exists.

__X__ Each such person, concern or organization is listed below.

NAME: University Technologies International Inc.

ADDRESS: 609 14th Street, NW, Suite 204

Calgary, Alberta, Canada T2N 2A1

____ Individual __X__ Small Business ____Nonprofit Organization

NAME: _____

ADDRESS: _____

_____

____ Individual ____ Small Business ____Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28(b)]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing: T.G. Falconer

Title in Organization: Vice-President, Administration

Winnipeg, Manitoba, Canada R3T 2N2

Signature: _____ **Date:** October 8, 1998

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

POWER ET AL.

Serial No.: Unknown

Filed: Herewith Examiner: Unassigned

For: ANTISENSE OLIGODEOXYNUCLEOTIDES REGULATING EXPRESSION OF TNF-ALPHA

Our File No.: 3045.00004

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Please amend the above-identified application prior to consideration of the application on the merits.

IN THE SPECIFICATION:

Page 1, line 11, in the "Cross Reference to Related Applications" section, after "60/062,718." insert --This application is a continuation application of U.S. Serial No. 08/176,862, filed October 22, 1998.--

IN THE CLAIMS:

Please cancel claims 1 and 2.

3. (Amended) A pharmaceutical or medical composition comprising as active ingredient at least one synthetic nuclease resistant antisense oligodeoxynucleotide [as set forth in claim 1] having a nucleotide

Attorney's Docket No. 3045.00002 PATENT

Applicant or Patentee: C. Bower and M. Mayne

Serial or Patent No: _____

Filed or Issued: _____

For: ANTISENSE OLIGODEOXYNUCLEOTIDES REGULATING EXPRESSION OF TNF-α

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)--SMALL BUSINESS CONCERN

I hereby declare that I am:

_____ the owner of the small business concern identified below:

__X__ an official of the small business concern empowered to act on behalf of the concern identified below:

Name of Concern: University Technologies International Inc.

Address of Concern: 609 14th Street NW, Suite 204

Calgary, Alberta, Canada T2N 2A1

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement: (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when, either directly or indirectly, one concern controls or has the power to control the other, or a third-party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention referenced above.

Described in:

__X__ the specification filed herewith.

_____ application referenced above.

_____ patent referenced above.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c), if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

* NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME: University of Manitoba

ADDRESS: Administration Building - Room 202

Winnipeg, Manitoba, CANADA R3T 2N2

___ Individual ___ Small Business _x_ Nonprofit Organization

NAME: _____

ADDRESS: _____

_____

___ Individual ___ Small Business ___ Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28(b)]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing: Beverley Sheridan

Title in Organization: President/Chief Executive Officer

Address of Person Signing: 609 14th Street NW, Suite 204

Calgary, Alberta, Canada T2N 2A1

SIGNATURE: _____ Date: Oct. 21/98

sequence selected from the group consisting of SEQ. ID No. 4 and SEQ. ID No. 6 in a physiologically acceptable carrier or diluent.

4. (Amended) The pharmaceutical composition [as set forth in claim 1] of a synthetic nuclease resistant antisense oligodeoxynucleotide comprising either SEQ. ID No. 4 or SEQ. ID No. 6 and at least one other non-control AS-ODN selected from Tables 1 and 2 wherein a percent inhibition is greater than 25%.

Claim 5, line 2, please delete "capable of" and insert therefor --for--.

7. (Amended) A pharmaceutical composition for selectively [modulating] regulating mammalian [tunor] tumor necrosis factor alpha in a mammal in need of such treatment consisting of

an effective amount of at least one active ingredient [as set forth in claim 1] a synthetic nuclease resistant antisense oligodeoxynucleotide having a nucleotide sequence selected from the group consisting of SEQ. ID No. 4 and SEQ. ID No. 6 in a pharmaceutically physiologically acceptable carrier or diluent.

13. (New) A method of selectively regulating mammalian tumor necrosis factor alpha by the steps of targeting for treatment the tumor necrosis factor alpha splice region and then specifically modify the region to regulate the mammalian tumor necrosis factor alpha.

14. (New) The method of claim 13 further including the step of administering an effective amount of a synthetic nuclease resistant antisense oligodeoxynucleotide which targets exon sequences flanking donor splice sites.

15. (New) A method of inhibiting tumor necrosis factor alpha by targeting for treatment the tumor necrosis factor alpha splice region.

16. (New) The method of claim 15 further including the step of administering an effective amount of a synthetic nuclease resistant antisense oligodeoxynucleotide which targets exon sequences flanking donor splice sites.

REMARKS

Claims 3-12 are currently pending in the application. Only claims 3, 5 and 9 are in independent form.

Claims 5 and 7 stand rejected under 35 U.S.C. Section 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention.

Specifically, the Office Action states that claim 5 recites the phrase "a synthetic nuclease resistant antisense oligodeoxynucleotide capable of selectively modulating". The term "capable" as recited in this claim is considered vague and indefinite. Accordingly, the term "capable" has been removed and instead this claim recited "a synthetic nuclease resistant antisense oligodeoxynucleotide for selectively modulating".

The Office Action also holds that claim 7 recites the term "tunor" and it is believed that this term is spelled improperly and should recite "tumor". Accordingly, this typographical error has been fixed to recite "tumor".

Claims 3-4 and 7-10 stand rejected under 35 U.S.C. Section 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

Specifically, the Office Action states that claims 3-4 and 7-10 are drawn to pharmaceutical compositions comprising a synthetic nuclease resistant antisense oligodeoxynucleotide, compositions comprising antisense oligodeoxynucleotides which selectively modulate human tumor necrosis factor alpha, and methods of modulating the expression of human tumor necrosis factor in a mammal. Further, it is stated that there are no general guidelines for the successful *in vivo* delivery of antisense/ribozyme compounds currently not in the art, nor are such guidelines provided in the specification as filed. *Crooke (1998)*, is cited for the conclusion that "extrapolations from in vitro uptake studies to predictions about in vivo pharmacokinetic behavior are entirely inappropriate". Therefore, the Office Action states that the specification does not describe the pharmaceutical compositions comprising antisense oligodeoxynucleotides targeting human tumor necrosis factor alpha, and methods of use of said compositions recited in these claims in a sufficient manner so as to enable one of ordinary skill in the art to practice the present invention without undue experimentation.

However, the method as cited in the present claims was tested *in vivo* in mice as presented in the attached article by Neuraths et al. This paper details using effective amounts of at least one active ingredient which is a synthetic nuclease resistant antisense oligodeoxynucleotide for regulating mammalian tumor necrosis factor alpha in a mammal. Accordingly, since the Neuraths et al. article utilizes the method set forth in the present application, there is sufficient detail present in the present application so as to enable one of ordinary skill in the art to practice the present invention without undue experimentation. Additionally, as shown by the attached articles by Neuraths et al., Bennett et al., Nyce et al., and Wojcik et al., there is increasing evidence showing that in vivo results can be shown based on the in vitro laboratory studies. These are all articles published prior to the priority date of the present application showing that it was known by those skilled in the art that the *in vitro* results of the present invention could be utilized to show the expected *in vivo*

results of such experimentation. Hence, undue experimentation is not required and the claims are enabled.

Claim 7 stands rejected under 35 U.S.C. Section 112, first paragraph, as containing subject matter which is not described in the specification in such a way as to reasonably convey one skilled in the relevant art the inventors, at the time the invention was filed, had possession of the claimed invention.

Specifically, the Office Action states that claim 7 reads on compositions comprising antisense oligodeoxynucleotides capable of selectively "modulating" mammalian tumor necrosis factor alpha. However, claim 7 has been amended to state "regulating" mammalian tumor necrosis factor alpha.

Claim 7, according to the Office Action, also recited a pharmaceutical composition for selectively modulating mammalian "tumor" necrosis factor alpha in a mammal. The specification as filed describes only a single class of mammalian tumor necrosis factor alpha, human tumor necrosis factor alpha. The Office Action states that the specification as filed does not provide any guidance or information of other mammalian tumor necrosis factor alpha mRNAs or proteins of other mammals that would one to predict the structure of these target molecules or potential target sites that would be susceptible to antisense inhibition. Therefore, the Office Action concludes that Applicants are not in possession of antisense oligodeoxynucleotides which modulate a human necrosis factor alpha gene from any other source than human. However, the application does provide a detailed analysis of the effect of the synthetic antisense oligodeoxynucleotides in murine cells. This is

established in the examples, specifically at pages 29-39 wherein the examples detail the effect of the AS-ODN treatment on murine macrophages. Accordingly, there is sufficient detail in the application to show that Applicants were in possession of antisense oligodeoxynucleotides which modulate a mammalian tumor necrosis factor alpha gene from sources other than humans at the time the application was filed.

Claims 5-12 stand rejected under 35 U.S.C. Section 112, first paragraph, because the specification, while being enabling for inhibition of an expression of human tumor necrosis factor alpha in vitro, does not reasonably provide enablement for inhibition of expression of human tumor necrosis factor alpha in vivo, nor does it provide enablement for "modulation" of expression of human tumor necrosis factor alpha, in vitro or in vivo. The Office Action states that the specification does not enable any person skilled in the art to which it pertains or with which it is most nearly connected, to practice the invention commensurate with the scope of the claims.

Specifically, the Office Action states that the claims are drawn to compositions comprising antisense oligodeoxynucleotides "capable of selectively modulating" human tumor necrosis factor alpha and methods of administering said compositions. If the scope of the claims are truly limited to antisense-based nucleic acid molecules, only inhibition is enabled, not modulation, since modulation implies both increasing and decreasing the expression or activity of a molecule. Therefore, since the specification does not teach an increase in expression and/or stability and since the state-of-the-art of antisense/ribozime teaches only inhibition, Applicants claim to "modulate" is not enabled to the extent that it reads on an antisense/ribozime based system.

However, as stated previously, the claims have been amended to state "regulation" which is defined on page 7 of the application lines 20-22 stating that regulation "it is meant that the expression of the TNF-ALPHA is inhibited or reduced by the action of the AS-ODNs thus indicating that only inhibition of the expression is claimed. Additionally, as stated previously, there is support for the use of the present method in vivo since there is knowledge in the art at the time the application was filed for the use of taking in vitro results and showing their use in vivo and showing the results which can be obtained in vivo. Further, as shown previously, the method of the present application has been conducted in vivo and has achieved the desired results as claimed in the presently pending independent claims. Accordingly, there is sufficient enablement in the specification for in vivo results and enablement for the term "regulation".

The remaining dependent claims not specifically discussed herein are ultimately dependent upon the independent claims. References as applied against these dependent claims do not make up for the deficiencies of those references as discussed above, the prior art references do not disclose the characterizing features of the independent claims discussed above. Hence, it is respectfully submitted that all of the pending claims are patentable over the prior art.

In view of the present amendment and foregoing remarks, reconsideration of the rejections and advancement of the case to issue are respectfully requested.

The Commissioner is authorized to charge any fee or credit any overpayment in connection with this communication to our Deposit Account No. 11-1449.

Respectfully submitted,

KOHN & ASSOCIATES

Kenneth I. Kohn
Registration No. 30,955
30500 Northwestern Highway
Suite 410
Farmington Hills, MI 48334
(248) 539-5050

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

Express Mail Mailing Label No.: EL 358 311 628 US
Date of Deposit: November 15, 1999

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office To Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington, DC 20231 BOX CONTINUATION APPLICATION.

Constance McLean

ANTISENSE OLIGODEOXYNUCLEOTIDES REGULATING EXPRESSION OF TNF-α

CROSSREFERENCE TO RELATED APPLICATIONS

This application claims priority from United States Provisional Application 60/062,718.

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

The present invention provides antisense oligodeoxynucleotides targeted to exon sequences flanking donor splice sites which regulate expression of TNF-α.

2. DESCRIPTION OF RELATED ART

There has been increasing interest in the development of antisense oligodeoxyribonucleotides (AS-ODNs hereinafter) as therapeutic agents and experimental tools (Stein and Cheng, 1993; Wagner, 1994). However, despite the improvement in affinity for target RNA, increased resistance to nucleolytic cleavage, and

enhanced delivery of AS-ODNs to cells and their nuclei (Hodges and Crooke, 1995), high concentrations of AS-ODNs continue to be required to inhibit gene expression. To some extent, high AS-ODN concentrations have hampered the development of this technology as an effective pharmacological agent because of cost and non-specific AS-ODN actions.

Many genes encode pre-mRNAs containing introns that are removed by a splicing process that is directed by a complex of small nuclear ribonucleic proteins (snRNPs) called the spliceosome (Staley and Guthrie, 1998). Several reports indicate that gene expression is effectively inhibited by AS-ODNs targeting the intron/exon boundaries of splice sites (Boeve and De Ley, 1994; Dominski and Kole, 1996; Dominski and Kole, 1994; Hodges and Crooke, 1995; Moulds et al., 1995), likely because these domains direct splicing events (Staley and Guthrie, 1998). It has previously been shown in cell free systems that the degree of sequence variability at splice sites influences splicing events (Dominski and Kole, 1994), suggesting that pre-mRNAs with variant splice site sequences would be ideal targets for AS-ODN treatment (Hodges and Crooke, 1995). Since exon sequences upstream of donor (5'), and downstream of acceptor (3') splice sites within pre-mRNA play a critical role in processing RNA (Staley and Guthrie,

1998), it is plausible that these sites encode RNA domains highly susceptible to AS-ODN-mediated inhibition of gene expression. To date, this hypothesis has not been tested systematically in a biologically relevant system such as tumor necrosis factor alpha (TNF-α) production in cell culture or *in vivo*.

Under normal conditions, TNF-α's expression is tightly regulated by rapid mRNA turnover (Gearing et al., 1995). However, in disease states, its expression is perturbed, resulting in overexpression (Sharief and Hentges, 1991; Tracey and Cerami, 1994). TNF-α is implicated in the pathogenesis of several inflammatory diseases including multiple sclerosis (MS) (French-Constant, 1994), rheumatoid arthritis (RA) (Lupia et al., 1996), viral infections such as human immunodeficiency virus (HIV) (Fauci, 1996) and, bacterial infections causing sepsis (Tomioka et al., 1996). TNF-α neutralizing antibodies (Givner et al., 1995), soluble TNF-α receptors (Moreland et al., 1997), or gene knockouts of the TNF receptor (p55) (Pfeffer et al., 1993) mitigate the harmful effects of TNF-α observed in several animal models of inflammation (Probert et al., 1995; Selmaj et al., 1991). However, these approaches do not limit TNF-α synthesis.

Several studies show that AS-ODNs targeting TNF-α mRNA limit TNF-α synthesis (Hartmann et al., 1996; Lefebvre d'Hellencourt et al., 1996; Rojanasakul et al., 1997; Taylor et al., 1996). However, in these reports, concentrations of AS-ODNs in excess of 2 μM, were required to achieve significant inhibition. High concentrations of AS-ODNs may induce non-specific inflammatory cell responses (Hartmann et al., 1996) as well as other non-specific effects (Gao et al., 1992; Khaled et al., 1996; Perez et al., 1994). Nevertheless, earlier reports suggest that expression of other genes can be regulated by low concentrations (≤ 1 μM) of AS-ODNs (Hanecak et al., 1996; Miraglia et al., 1996). Therefore it would be useful to develop AS-ODNs that can be used in low concentrations to regulate TNF-α production in inflammatory responses.

SUMMARY OF THE INVENTION

According to the present invention, a synthetic nuclease resistant antisense oligodeoxynucleotide capable of selectively modulating expression of human tumor necrosis factor-alpha by targeting exon sequences flanking donor splice sites, thereby regulating

expression of TNF-α in a patient in need of such therapy is provided. In an embodiment either AS-ODN having the sequence set forth in SEQ ID No:4 or SEQ ID No:6 or a combination thereof can be used. The AS-ODN is administered either as the active ingredient in a pharmaceutical composition or by utilizing gene therapy techniques as an expression vector.

DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

FIGURE 1 is a bar graph which shows AS-ODNs targeting exon sequences flanking the donor splice site of exon 2 and 3 of TNF-α effectively inhibit TNF-α protein production. Percent reduction of TNF-α levels in PMA/PHA stimulated U937 cells treated with AS-ODNs targeting various domains within the TNF-α open reading frame. PMA/PHA stimulated U937 cells produced 750 ± 75 pg TNF-α/ml/million cells. * ($p \leq 0.01$) ** ($p \leq 0.001$). **Unstim**- Unstimulated U937 cells; **O-1 through O-21**- ODNs

complementary to different sequences of TNF-α. Data are presented as a mean ± SD (n=3).

FIGURE. 2A-B are bar graphs which show ORF4 (SEQ ID No:4) and ORF6 (SEQ ID No:6) reduce TNF-α production in a dose-dependent manner. (FIGURE 2*A*) U937 cells were treated with ORF4 or ORF6 (1 μM, 100 and 10 nM) and supernatant TNF-α levels were measured by ELISA. (FIGURE 2*B*) AS-ODNs are not cytotoxic to U937 cells. Data are presented as a mean ± SD (n=3). * ($p \leq 0.01$) ** ($p \leq 0.001$).

FIGURE 3 is a bar graph which shows ORF4-PE dose-dependently reduces TNF-α mRNA in stimulated U937 cells. RT-PCR was used to detect TNF-α and GAPDH mRNA levels in U937 cells treated with ORF4-PE. Densiometric analysis of TNF-α RT-PCR products from U937 cells treated with ORF4-PE. Relative TNF-α mRNA levels were calculated based on the pixel density ratio of TNF-α:GAPDH PCR product in each separate reaction. Data are presented as a mean ± SD (n=3). * ($p \leq 0.05$) ** ($p \leq 0.01$).

FIGURE 4A-B are bar graphs which show ORF4-PE specificity for TNF-α. (FIGURE 4*A*) U937 cells were treated with ORF4-PE and supernatant TNF-α and IL-6 levels were measured by ELISA. (*FIGURE 4B*) ORF4-PE-mediated inhibition of mitogen-induced TNF-α gene

expression in primary human PBMC, macrophages and murine monocytes. PMA/PHA stimulated PBMC produced 1250 ± 110 pg TNF-α/ml/million cells. LPS-stimulated primary macrophages produced 13,500 ± 1,700 pg TNF-α/ml/million cells. LPS-stimulated murine monocytes produced 7,100 ± 875 pg TNF-α/ml/million cells. Data are presented as mean ± SD (n=3). * ($p \leq 0.01$) ** ($p \leq 0.001$).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a synthetic nuclease resistant antisense oligodeoxynucleotide (AS-ODN) capable of selectively modulating human tumor necrosis factor alpha (TNF-α) by targeting exon sequences flanking donor splice sites thereby regulating expression of TNF-α in a patient in need of such treatment. Donor splice sites represent the 3' end of an exon and are located at the junction between the exon and intron.

By modulating/regulating it is meant that the expression of TNF-α is inhibited or reduced by the action of the AS-ODNs.

In an embodiment either:

SEQ ID No:4 CTG ACT GCC TGG GCC AGA GGG CTG ATT AG

SEQ ID No:6 CCA CAT GGG CTA CAG GCT TGT CAC TCG

can be used or any combination thereof.

As shown in the Example hereinbelow, SEQ ID No:4, when made nuclease resistant by phosphorothioate bonds linking between the four 3'-terminus nucleotide bases, is effective and non-toxic.

Since exon sequences are critical in mRNA processing (Dominski and Kole, 1996; Dominski and Kole, 1994) and genes with short internal exons, such as TNF-α, are highly susceptible to exon skipping (Dominski and Kole, 1991), as shown herein it appears that TNF-α's internal exons encode domain is highly susceptible to AS-ODN actions. As shown herein: (a) the inhibitory action of AS-ODNs targeting TNF-α exon sequences upstream of donor sites and downstream of acceptor sites as well as AS-ODNs that target other regions of the TNF-α gene were determined; (b) stringent criteria in the design and selection of each AS-ODN was used for maximizing its potential efficiency and; (c) AS-ODNs developed by the above criteria are shown effective in different cell types in which TNF-α was stimulated by different signaling pathways. AS-ODNs targeting exon sequences flanking the 2nd or 3rd exon donor splice sites

significantly inhibited TNF-α protein production. Therefor exon sequences flanking donor splice sites of the small internal exons of TNF-α are domains that are highly susceptible to the AS-ODN treatment of the present invention.

The present invention provides pharmaceutical compositions as described hereinbelow and gene therapy means of administering the AS-ODN of the present invention to regulate TNF-α expression. The active ingredient of the pharmaceutical composition is at least one synthetic nuclease resistant antisense oligodeoxynucleotides, or ribozymes, targeting exon sequences flanking donor splice sites, such as SEQ ID No:4 OR SEQ ID No:6 in a physiologically acceptable carrier or diluent. The concentration range of the AS-ODN in the pharmaceutical composition is generally 1.0 μM to 100nM.

Phosphorothioate antisense oligonucleotides do not normally show significant toxicity and exhibit sufficient pharmacodynamic half-lives in animals [Agarwal *et al.*, 1996]. Antisense induced loss-of-function phenotypes related with cellular development were shown for the glial fibrillary acidic protein (GFAP), for the establishment of tectal plate formation in chick and for the N-myc protein, responsible for the maintenance of

cellular heterogeneity in neuroectodermal cultures (ephithelial vs. neuroblastic cells, which differ in their colony forming abilities, tumorigenicity and adherence). Antisense oligonucleotide inhibition of a basic fibroblast growth factor (bFgF), having mitogenic and angiogenic properties, suppressed 80% of growth in glioma cells [Morrison, 1991] in a saturable and specific manner. Being hydrophobic, AS-ODN interact well with phospholipid membranes [Akhter *et al.*, 1991]. Following their interaction with the cellular plasma membrane, they are actively (or passively) transported into living cells [Loke *et al.*, 1989]

The term "oligodeoxynucleotide" refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages. The term also includes modified or substituted oligomers comprising non-naturally occurring monomers or portions thereof, which function similarly. Incorporation of substituted oligomers is based on factors including enhanced cellular uptake, or increased nuclease resistance and are chosen as is known in the art. The entire oligodeoxynucleotide or portions thereof may contain the substituted oligomers.

Instead of an antisense sequence, as discussed herein above, ribozymes may be utilized for suppression of gene function. This is particularly necessary in cases where antisense therapy is limited by stoichiometric considerations [Sarver et al., 1990, Gene Regulation and Aids, pp. 305-325]. Ribozymes can then be used that will target the same sequence. Ribozymes are RNA molecules that possess RNA catalytic ability [see Cech for review] and cleave a specific site in a target RNA. The number of RNA molecules that are cleaved by a ribozyme is greater than the number predicted by stochiochemistry. [Hampel and Tritz, 1989; Uhlenbeck, 1987]. Therefore, the present invention also allows for the use of the ribozyme sequences, targeted to exon sequences flanking donor splice sites, which regulate expression of TNF-α expression and contain the appropriate catalytic center. The ribozymes are made and delivered as discussed herein below. The ribozymes may be used in combination with the antisense sequences.

Ribozymes catalyze the phosphodiester bond cleavage of RNA. Several ribozyme structural families have been identified including Group I introns, RNase P, the hepatitis delta virus ribozyme, hammerhead ribozymes and the hairpin ribozyme originally derived from the negative strand of the tobacco ringspot virus satellite RNA

(sTRSV) (Sullivan, 1994; U.S. Patent No. 5,225,347, columns 4-5). The latter two families are derived from viroids and virusoids, in which the ribozyme is believed to separate monomers from oligomers created during rolling circle replication. Hammerhead and hairpin ribozyme motifs are most commonly adapted for trans-cleavage of mRNAs for gene therapy (Sullivan, 1994). The ribozyme type utilized in the present invention is selected as is known in the art. Hairpin ribozymes are now in clinical trial and are the preferred type. In general, the ribozyme is from 20-100 nucleotides in length.

Nuclease resistance, where needed, is provided by any method known in the art that does not substantially interfere with biological activity of the antisense oligodeoxynucleotides or ribozymes as needed for the method of use and delivery [Iyer et al., 1990; Radhakrishnan, et al., 1990; Eckstein, 1985; Spitzer and Eckstein, 1988; Woolf et al., 1990; Shaw et al., 1991]. As shown herein in the Example, ORF4-PE (a phosphorothioate derivative of ORF4; SEQ ID No:4) is a preferred embodiment.

Modifications that can be made to antisense oligonucleotides and ribozymes in order to enhance nuclease resistance include modifying the phosphorous or oxygen heteroatom in the phosphate backbone, short chain

alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. These include preparing methyl phosphonates, phosphorothioates, phosphorodithioates and morpholino oligomers. In one embodiment, it is provided by having phosphorothioate bonds linking some or all the nucleotide bases. Phosphorothioate antisense oligonucleotides do not normally show significant toxicity at concentrations that are effective and exhibit sufficient pharmacodynamic half-lives in animals [Agarwal *et al.*, 1996] and are nuclease resistant. Other modifications known in the art may be used where the biological activity is retained, but the stability to nucleases is substantially increased. The efficiency of inhibition and toxicity can be tested as shown herein in the Example to determine the most effective nuclease resistant protocol.

The nuclease resistant AS-ODNs of the present invention are administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex, body weight and other factors known to medical practitioners. The pharmaceutically "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must

be effective to achieve improvement including but not limited to improved survival rate or more rapid recovery, lower levels of expressed mRNA for TNF-α or improvement or elimination of symptoms and other indicators as are selected as appropriate measures by those skilled in the art. General methods of administration are provided herein which can be modified as known in the art to accommodate the requirements of maintaining and delivery of AS-ODNs.

Once the nuclease resistant oligonucleotide sequences are ready for delivery they can be introduced into cells, as is known in the art. Transfection, electroporation, fusion, liposomes, colloidal polymeric particles and viral vectors, as well as other means known in the art, may be used to deliver the oligonucleotide sequences to the cell. The selected method depends on the cells to be treated and the location of the cells and will be known to those skilled in the art. Localization can be achieved by liposomes, having specific markers on the surface for directing the liposome, by having injection directly into the tissue containing the target cells, by having depot associated in spatial proximity with the target cells, specific receptor mediated uptake, viral vectors, or the like. Transfection vehicles such as liposomes can also be used to introduce the non-viral

vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

The pharmaceutical composition of the present invention may be a combination of the AS-ODNs provided in the present invention. The combination is assembled and dosed as is known in the art. Further, the composition of the present invention may be a combination of one of the AS-ODNs provided in the present invention in combination with at least one other non-control AS-ODN selected from Table 1 or Table 2. This combination would have a low toxicity with a percent inhibition of approximately 25% or more.

The patient being treated is a warm-blooded animal and, in particular, mammals including man. The pharmaceutically acceptable carriers, diluents, adjuvants and vehicles, as well as implant carriers generally refer to inert, non-toxic solid or liquid fillers, diluents or encapsulating material not reacting with the active ingredients of the invention.

It is noted that humans are treated generally longer than the cells exemplified herein which treatment has a length proportional to the length of the disease process and drug effectiveness.

The AS-ODN of the present invention can be administered utilizing gene therapy techniques. Generally, a DNA expression vector comprising an expressible promotor/transcriptional initiator and the AS-ODN sequence is utilized.

"By gene therapy" as used herein refers to the transfer of genetic material (e.g DNA or RNA) of interest into a host to treat or prevent a genetic or acquired disease or condition phenotype. The genetic material of interest encodes a product (e.g. a protein, polypeptide, peptide, functional RNA, antisense) whose production *in vivo* is desired. For example, the genetic material of interest can encode a hormone, receptor, enzyme, polypeptide or peptide of therapeutic value. Alternatively, the genetic material of interest encodes a suicide gene. For a review see, in general, the text "Gene Therapy" (Advances in Pharmacology 40, Academic Press, 1997).

Two basic approaches to gene therapy have evolved: (1) *ex vivo* and (2) *in vivo* gene therapy. In *ex vivo* gene therapy cells are removed from a patient, and while being cultured are treated *in vitro*. Generally, a functional replacement gene is introduced into the cell via an appropriate gene delivery vehicle/method (transfection, transduction, homologous recombination,

etc.) and an expression system as needed and then the modified cells are expanded in culture and returned to the host/patient. These genetically reimplanted cells have been shown to express the transfected genetic material *in situ*.

In *in vivo* gene therapy, target cells are not removed from the subject rather the genetic material to be transferred is introduced into the cells of the recipient organism *in situ*, that is within the recipient. In an alternative embodiment, if the host gene is defective, the gene is repaired *in situ* [Culver, 1998]. These genetically altered cells have been shown to express the transfected genetic material *in situ*.

The gene expression vehicle is capable of delivery/transfer of heterologous nucleic acid into a host cell. The expression vehicle may include elements to control targeting, expression and transcription of the nucleic acid in a cell selective manner as is known in the art.

The expression vehicle can include a promotor for controlling transcription of the heterologous material and can be either a constitutive or inducible promotor to allow selective transcription. Enhancers that may be required to obtain necessary transcription levels can optionally be included. Enhancers are generally any non-

translated DNA sequence which works contiguously with the coding sequence (in cis) to change the basal transcription level dictated by the promoter. The expression vehicle can also include a selection gene.

Vectors can be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods can be found generally described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989), Chang et al., *Somatic Gene Therapy*, CRC Press, Ann Arbor, MI (1995), Vega et al., *Gene Targeting*, CRC Press, Ann Arbor, MI (1995), *Vectors: A Survey of Molecular Cloning Vectors and Their Uses*, Butterworths, Boston MA (1988) and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see United States patent 4,866,042 for vectors involving the central nervous system and also United States patents 5,464,764 and 5,487,992 for positive-negative selection methods.

The recombinant vector can be administered in several ways. If viral vectors are used, for example, the procedure can take advantage of their target specificity and consequently, do not have to be

administered locally at the diseased site. However, local administration can provide a quicker and more effective treatment, administration can also be performed by, for example, intravenous or subcutaneous injection into the subject. Injection of the viral vectors into a spinal fluid can also be used as a mode of administration, especially in the case of neuro-degenerative diseases. Following injection, the viral vectors will circulate until they recognize host cells with the appropriate target specificity for infection.

An alternate mode of administration can be by direct inoculation locally at the site of the disease or pathological condition or by inoculation into the vascular system supplying the site with nutrients or into the spinal fluid. Local administration is advantageous because there is no dilution effect and, therefore, a smaller dose is required to achieve expression in a majority of the targeted cells. Additionally, local inoculation can alleviate the targeting requirement required with other forms of administration since a vector can be used that infects all cells in the inoculated area. If expression is desired in only a specific subset of cells within the inoculated area, then promoter and regulatory elements that are specific for the desired subset can be used to accomplish this goal. Such non-targeting vectors can be, for example, viral

vectors, viral genome, plasmids, phagemids and the like. Transfection vehicles such as liposomes and colloidal polymeric particles can also be used to introduce the non-viral vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

Many studies show that small incremental differences in TNF-α protein levels have large effects on a variety of biological processes including viral replication (Fauci, 1996), physiological and pathological cell responses to infectious diseases (Beutler and Grau, 1993), cell death (Beutler and van Huffel, 1994; Probert et al., 1997; Talley et al., 1995), and normal cell growth and development (Arvin et al., 1996; Beutler and Grau, 1993; Tracey and Cerami, 1994). Given TNF-α's pivotal role in disease and normal development, complete interruption of TNF-α expression is not desirable. Thus, molecular tools, such as AS-ODNs, which modulate, as opposed to eliminate gene expression, provide optimal gene regulation.

As show herein in the Example, ORF4-PE (a phosphorothioate derivative of ORF4; SEQ ID No:4) significantly reduces TNF-α mRNA levels by greater than 80% and protein levels by approximately 60% in stimulated U937 cells. A greater reduction of TNF-α mRNA compared

to protein levels is not unexpected as TNF-α has a short half-life and thus, rapid mRNA turnover (Zheng and Specter, 1996). ORF4-PE was sequence specific, efficacious in different cell types, under different stimulatory conditions and did not influence the gene expression of another proinflammatory cytokine, IL-6. Further study showed that ORF4-PE, alone, does not induce TNF-α expression in U937 or PBMC (data not shown), likely due to the lack of CpG moieties and G quartets which encode domains that may stimulate immune cells (Hartmann et al., 1996; Krieg et al., 1997; Krieg et al., 1996). Thus, the present invention which provides for efficient regulation of TNF-α gene expression can be achieved by using ODNs targeting exon sequences flanking donor sites.

Several reports show a reduction of TNF-α levels *in vitro* using either an antisense approach (Arima et al., 1997; Hartmann et al., 1996; Lefebvre d'Hellencourt et al., 1996; Liang et al., 1996; Rojanasakul et al., 1997; Taylor et al., 1996; Yang et al., 1993) or a formation of triplex DNA complexes (Aggarwal et al., 1996). However, in these studies, AS-ODN concentrations ranging from 2-20 μM were required to inhibit TNF-α expression. Most studies targeting the highly conserved AUG sequence of TNF-α, assumed that this domain is the most efficient site for interruption of translation. Indeed, this

approach indicates that antisense molecules directed at the 5' start region, when efficiently delivered to cells, can effectively reduce TNF-α levels (Rojanasakul et al., 1997). However, a comparison of ORF4-PE with the most efficient TNF-α-specific ODNs reported to date, which targeted the 5' AUG start region (Hartmann et al., 1996; Rojanasakul et al., 1997), showed that ORF4-PE was unexpectedly approximately 2.5-fold more efficacious at reducing TNF-α levels (Table 2). An AS-ODN complementary to the 5' AUG region of TNF-α (ORF1; SEQ ID No:1) was equally inefficient at reducing TNF-α levels (Fig. 1), suggesting that under these experimental conditions, exon sequences upstream of donor splice sites are domains that are highly vulnerable to ODN actions.

The mechanism by which ORF4-PE significantly inhibits TNF-α levels is unclear. ORF4-PE, however, is 100% complementary to the exon sequence (exon #2) flanking the donor splice site and thus, may hybridize with higher efficiency to the exon sequence, compared to the U1 small nuclear ribonucleic acid (snRNA), thereby competitively interrupting spliceosome formation and subsequent splicing (Staley and Guthrie, 1998). An examination of the upstream nucleotide sequence flanking the donor splice site of exon #2 reveals a non-consensus, variant sequence. Encoded within the exon immediately

upstream of the donor splice site (3' end of the 2nd exon) is the sequence 5'TCA3' whereas the sequence 5'A/C AG3' more frequently occurs at approximately 70, 62 and 80% respectively, at these positions (Hertel et al., 1997; Padgett et al., 1986; Tarn and Steitz, 1997). This same position within the third exon of TNF-α however, is fully conserved which may explain why ORF6, which targets the exon sequence upstream of the donor site flanking exon #3, is less effective than ORF4.

Failure to recognize short internal exons by the spliceosome may be due to juxtaposition of adjacent 3' and 5' splice sites of internal exons, thereby creating steric hindrance and improper spliceosome/splice site interactions (Dominski and Kole, 1991). Both internal exons of TNF-α (exon #2- 46 bp and exon #3- 48 bp) (accession # M16441: Genbank) are less than 50 bp and thus, may be subject to exon skipping (Dominski and Kole, 1991). Given the rarity of short internal exons in eukaryotic genes (less than 4%) (Hawkins, 1988) and their susceptibility to exon skipping, the addition of competing AS-ODNs would further impede efficient mRNA splicing. In addition, exon skipping can be induced by improper recognition of weak donor splice sites (Dominski and Kole, 1991). Indeed, encoded within the second exon flanking the donor splice site of human TNF-α is a

variant sequence that may be competitively inhibited by ORF4-PE. In either event, the exon skipping or direct inhibition of splicing would lead to decreased levels of TNF-α mRNA.

An alternative possibility is that ORF4-PE hybridizes with high affinity to processed mRNA, activating RNase H (Wagner, 1994). Mfold RNA modeling (Jacobson and Zuker, 1993) of human TNF-α mRNA (data not shown) however, shows that the domain complementary to ORF4-PE is predicted to exist as a double stranded structure. Previous studies indicate that double-stranded nucleic acid structures do not provide highly stable targets for antisense AS-ODNs (Lima et al., 1992; Thierry et al., 1993). Thus, although secondary structure of mRNA may limit AS-ODN-mediated actions (Laptev et al., 1994; Mishra et al., 1996; Mishra and Toulme, 1994), it is unexpected to find that ORF4-PE is binding to processed TNF-α mRNA and therefore activating RNase H.

Finally, although Lipofectin was found to decrease TNF-α production in U937 cells by approximately 15%, use of this carrier significantly increased the efficiency of ORF4, ORF4-PE and ORF6. These studies indicated that all AS-ODNs required a final positive net charge (as determined by the ratio of Lipofectin to ODN (Lappalainen et al., 1997) in order to effectively reduce gene

expression. Specifically, Lipofectin concentrations of 10, 5 and 1 μg/ml were mixed with ORF4 (1 μM) with 10 μg of Lipofectin having the highest degree of efficiency (data not shown). These findings are similar to previous reports of Lipofectin-enhanced cellular uptake of AS-ODNs (Hartmann et al., 1996; Lappalainen et al., 1997; Zelphati and Szoka, 1996).

These studies demonstrate that exon sequences upstream of donor splice sites within small internal exons of a naturally occurring gene, constitute domains that are highly susceptible to AS-ODN-mediated inhibition of gene expression. In addition, a highly efficient antisense AS-ODN, ORF4-PE (SEQ ID No:4), was designed which is useful *in vitro* and *in vivo* in models which study TNF-α dysregulation as well as therapeutically.

The above discussion provides a factual basis for the use of AS-ODNs that can be used in low concentrations to regulate TNF-α production in inflammatory responses. The methods used with and the utility of the present invention can be shown by the following non-limiting examples and accompanying Figures and Tables.

EXAMPLES

METHODS:

General methods in molecular biology: Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York (1989), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989) and in Perbal, *A Practical Guide to Molecular Cloning*, John Wiley & Sons, New York (1988), and in Watson et al., *Recombinant DNA*, Scientific American Books, New York and in Birren et al (eds) *Genome Analysis: A Laboratory Manual Series, Vols. 1-4* Cold Spring Harbor Laboratory Press, New York (1998) and methodology as set forth in United States patents 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057 and incorporated herein by reference. Polymerase chain reaction (PCR) was carried out generally as in *PCR Protocols: A Guide To Methods And Applications*, Academic Press, San Diego, CA (1990). In-situ (In-cell) PCR in combination with Flow Cytometry can be used for detection of cells containing specific DNA and mRNA sequences (Testoni et al, 1996, Blood 87:3822.)

General methods in immunology: Standard methods in immunology known in the art and not specifically described are generally followed as in Stites et al.(eds), Basic and Clinical Immunology (8th Edition), Appleton & Lange, Norwalk, CT (1994) and Mishell and Shiigi (eds), Selected Methods in Cellular Immunology, W.H. Freeman and Co., New York (1980).

Immunoassays In general, ELISAs as described herein are employed to assess the TNF-α levels. ELISA assays are well known to those skilled in the art. Both polyclonal and monoclonal antibodies can be used in the assays. Where appropriate other immunoassays, such as radioimmunoassays (RIA) can be used as are known to those in the art. Available immunoassays are extensively described in the patent and scientific literature. See, for example, United States patents 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521 as well as Sambrook et al, *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor, New York, 1989

Cell culture and primary cell preparations. The human promonocytic cell line, U937 was obtained from American Type Culture Collection, (ATCC CRL 1593.2; batch F12641) (12301 Parklawn Drive, Rockville, MD 20852, USA)

and was cultured as previously outlined (Chen et al., 1997). Murine monocyte cells, IC-21 (ATCC TIB 186) were maintained at an approximate density of 2.0 X 10^5 cells/ml in 10 mM HEPES buffered, RPMI media supplemented with 10% FBS. Human peripheral blood mononuclear cells (PBMC) and macrophages were purified from whole blood obtained from healthy volunteers as previously outlined (Power et al., 1995). Briefly, to obtain primary human macrophages, PBMC were cultured for 3 d. in RPMI 1640 media supplemented with 20% heat-inactivated FBS and 50 units of penicillin and 50 μg of streptomycin/L. Non-adhering cells were removed by washing with RPMI media and adhering monocytic cells were removed and cultured at a density of 2.0 X 10^5 cells/ml for seven days in RPMI supplemented with 20% FBS and antibiotics. All cells were maintained at 37°C in a humidified growth chamber supplemented with 5% CO_2.

Design and synthesis of AS-ODNs. AS-ODNs were generated using the computer DNA modeling program PrimerSelect (DNASTAR) which designs AS-ODNs based on optimal free energy (Δ G), low dimer formation, low hairpin formation and high thermal stability. AS-ODNs were designed to target exon sequences upstream of the donor (5') and downstream of the acceptor (3') splice sites and other regions throughout the open reading frame

(ORF) of human TNF-α (accession # M10988: GenBank) and were assessed for sequence similarity with other non-TNF-α mRNAs (GenBank). TNF-α mRNA numbering was defined such that A in the AUG start codon was position +1. AS-ODNs were chosen if they met the following requirements: size between 16 and 30 mer; low hairpin formation (≤ 3 base pairs); low dimer formation (≤ 3 consecutive bases) and melting temperatures above 45 °C. All AS-ODNs were synthesized using standard phosphoramidite methods at 0.05 or 0.2 μmol scales and were HPLC purified by the manufacturer (Life Technologies, Mississauga, ONT).

Cell culture stimulation and AS-ODN treatment. Immortalized monocytic cells (U937 (human) or IC-21 (murine)) were cultured to approximately 80% confluency and were seeded at a density of 400,000 cells/ml (U937) or 200,000 cells/ml (IC-21) in a 96 well plate in OPTI-MEM serum reduced media (pH 7.4) supplemented with 5% FBS without antibiotics. Human PBMC were seeded at a density of 500,000 cells/ml. Serum levels were reduced to 5% as recommended for Lipofectin use by the manufacturer. AS-ODNs (1 μM, 100 and 10 nM) were mixed with Lipofectin (10μg/ml) (Life Technologies, Mississauga, Ontario, Canada) and added to the cell cultures for three hours. The cells were subsequently stimulated for 1 h with 10 ng/ml of phorbol-12-myristate-13-acetate (PMA) and 5

μg/ml phytohemagglutinin (PHA). The cells were then washed once with OPTI-MEM media supplemented with 5% FBS. AS-ODN/Lipofectin mixtures equal to the initial dosage were then added to each respective well. Since stimulated macrophages display maximum TNF-α mRNA levels at 3 hours, and protein formation at 3 to 4 hours (Zheng and Specter, 1996), all cells treated with AS-ODNs were incubated for 4 hours at 37 °C and supernatants were collected, centrifuged at 700 x g for 5 minutes and analyzed for TNF-α by ELISA. To determine AS-ODN cytotoxicity, U937 cells were treated with 5, 1, and 0.1 μM of AS-ODNs. AS-ODN-mediated cell death was determined both in the presence and absence of Lipofectin by measuring cell proliferation and viability (as measured by trypan blue exclusion) at 4, 8, and 24 hours. All antisense screening experiments were performed in triplicate, a minimum of three times.

LPS treatment of primary macrophages. Primary macrophage cultures (200,000 cells/ml) were maintained in complete RPMI media supplemented with 20% FBS and antibiotics for 7 days following purification. Cell cultures were washed once with RPMI media and then suspended in OPTI-MEM serum-reduced media supplemented with 5% FBS the day prior to the experiment. Macrophage cultures were treated with Lipofectin-delivered AS-ODNs

for 3 hours, washed once with OPTI-MEM media supplemented with 5% FBS and stimulated for 1 hour with 1 μg/ml lipopolysaccharide (LPS) (*E. coli* type 055:B5) (Sigma) and AS-ODNs were administered as outlined above. Following 4 hours incubation, the supernatants were collected and analyzed by ELISA.

Quantitative immunoassay for cytokines. TNF-α levels in tissue culture supernatants were determined by a sandwich ELISA as previously reported (Chen et al., 1997). Human IL-6 and murine TNF-α levels were quantified using sandwich ELISA according to the manufacturer (Pharmingen). Serial doubling dilutions of human or murine recombinant TNF-α (1250 to 4.5 pg/ml) or IL-6 (2500 to 19.5 pg/ml) was used to generate standard curves. For all experiments, values are corrected for the presence of Lipofectin and are presented as mean ± SD (n=3).

Total RNA extraction and RT-PCR. Total cellular RNA was prepared from approximately 1 X 10^6 cells as previously described (Gough, 1988). Pilot studies were performed comparing different PCR cycle number and input RNA concentrations to ensure linear amplification of template occurred. RT-PCR amplification was within linear range when 2 μg of total RNA was reverse transcribed into cDNA (Pharmacia, Mississauga, ONT)

followed by PCR amplification of 2 μl of cDNA product using either TNF-α or GAPDH cDNA specific primers (Chen et al., 1997; Wesselingh et al., 1993) for 25 cycles at 95°C denaturation (60 s), 60°C annealing (60 s) and 72 °C extension (60 s). These conditions confirmed previous findings by applicants (Chen et al., 1997; Wesselingh et al., 1993). Products were separated by agarose gel electrophoresis (1.4%), transferred to a nylon membrane and probed using a randomly labeled ^{32}P-dCTP human TNF-α cDNA (Wang et al., 1985) or human GAPDH (Chen et al., 1997). Densiometric analysis of RT-PCR products was performed using the public domain program NIH Image (Ver 1.60).

Statistical analysis. Results were statistically analyzed by two-tailed Student's *t*-test.

RESULTS

Exon sequences immediately upstream of donor splice sites of TNF-α are highly susceptible to AS-ODN-mediated inhibition. Recent evidence suggests that TNF-α production can be regulated by antisense AS-ODNs, however, AS-ODN concentrations ranging from 2-20 μM are required to reduce TNF-α levels in cells (Hartmann et al., 1996; Lefebvre d'Hellencourt et al., 1996; Rojanasakul et al., 1997; Taylor et al., 1996). At a concentration of 1 μM or less, applicants examined the

efficiency of AS-ODNs targeting exon sequences upstream of donor sites and downstream of acceptor splice sites as well as other regions within the TNF-α mRNA (Table 1). These studies unexpectedly show that AS-ODNs targeting exon sequences upstream of the donor splice site of exon 2 (ORF4; SEQ ID No:4) and 3 (ORF6; SEQ ID No:6) reduced TNF-α levels in PMA/PHA stimulated U937 cells (Fig. 1, Table 1). TNF-α levels in stimulated U937 cells were reduced by 62 ± 7% ($p<0.001$) by ORF4 (SEQ ID No:4) and 51 ± 9% ($p<0.005$) by ORF6 (SEQ ID No:6) (Fig. 1, Table 1). In contrast, AS-ODNs targeting exon sequences downstream of the acceptor sites of exon 2 (ORF3; SEQ ID No:3) and 3 (ORF5; SEQ ID No:5) or AS-ODNs targeting other TNF-α mRNA domains, including the 3' UTR UA rich region did not significantly reduce TNF-α levels (Fig. 1, Tables 1, 2). AS-ODNs designed to complement the 5' AUG start site of human TNF-α (Rojanasakul et al., 1997) were not as efficacious as ORF4 (SEQ ID No:4) or ORF6 (SEQ ID No:6) under the same conditions (Fig. 1, Table 1) and mismatched versions of ORF4 (n=2) did not significantly inhibit TNF-α production (Table 1). In addition, a 21 mer AS-ODN, O-8433 (SEQ ID No:23) that targets the HIV-1 *tat* gene, was used to assess for non-specific AS-ODN effects. O-8433 did not significantly affect supernatant TNF-α levels in stimulated U937 cells (Table 1).

Since ORF4 and ORF6 displayed the greatest inhibition of TNF-α synthesis, these antisense oligonucleotide molecules were further analyzed. In addition to 1 μM of ORF4 and ORF6 significantly reducing TNF-α levels, 100 and 10 nM of ORF4 reduced levels by 40 ± 9% and 19 ± 7% respectively and 100 and 10 nM of ORF6 reduced TNF-α levels by 26 ± 8% and 18 ± 9% respectively (Fig. 2a).

To ensure that 1 μM concentrations of AS-ODN were not toxic to U937 cells, AS-ODN concentrations as high as 5 μM were added to U937 cells which were subsequently tested for proliferation and viability. Five μM ORF4 had no effect on cell proliferation or viability at 4, 8 or 24 hours (Fig. 2b). Cells treated with AS-ODNs delivered by Lipofectin showed similar results up to 24 hours. After 24 hours treatment however, Lipofectin-treated cells showed significant cell death, presumable due to Lipofectin cytotoxicity (Bell et al., 1998; Yagi et al., 1993) (data not shown).

Detailed analysis of AS-ODNs targeting exon sequences flanking donor and acceptor splice sites. Since AS-ODNs complementary to the exon sequences upstream of the donor splice site of exons 2 and 3 of TNF-α significantly inhibited TNF-α production, adjacent nucleic acid domains

of the exons were examined in greater detail (Table 2). Specifically, AS-ODNs (n=10) were designed to target regions spanning the small internal exons (exons 2 and 3) of human TNF-α. All AS-ODNs were partially phosphorothioated (approximately 30%) and ORF4 (SEQ ID No:4) was partially phosphorothioated (ORF4-PR) or phosphorothioated at 3 bases on each end of ORF4 (SEQ ID No:4, ORF4-PE) in order to increase nuclease stability (Table 1) (Uhlmann et al., 1997). Only AS-ODNs targeting exon sequences upstream of the donor splice site, independent of their size, significantly reduced TNF-α production (Table 2). Conversely, AS-ODNs targeting the downstream exon sequences of the acceptor site did not reduce TNF-α levels to the same extent. Of all AS-ODNs tested in U937 cells, ORF4-PE was the most efficacious (65 ± 5%) (Table 2). In contrast, ORF4-PR, which was randomly phosphorothioated throughout its 29 mer sequence, reduced TNF-α levels by 42 ± 5% which was significantly less ($p<0.001$) than ORF4-PE (Table 2) suggesting that site(s) of phosphorothioation may be critical determinants of AS-ODN efficiency.

ORF4-PE (SEQ ID No:4) dose-dependently reduces TNF-α mRNA levels in stimulated U937 cells. To determine the extent to which ORF4-PE influenced TNF-α mRNA levels RT-PCR was

performed using primers that amplified a segment of TNF-α spanning exons 2, 3 and 4. RT-PCR products, confirmed by Southern analysis, showed that ORF4-PE dose-dependently reduced the levels of the correctly processed TNF-α mRNA. Densiometric analysis of RT-PCR products confirmed that as the concentration of ORF4-PE increased, TNF-α mRNA detection decreased (Fig. 3). Interestingly, an additional RT-PCR product, smaller than expected (430 bp), was present in samples treated with 1 μM ORF4-PE (data not shown). Although the source of this PCR product is unknown, this band may be the result of a cryptic splicing event (Hodges and Crooke, 1995). However, larger intermediary RNA species were not observed, perhaps due to rapid degradation of improperly spliced pre-mRNA (Khoury et al., 1979).

ORF4-PE (SEQ ID No:4) efficiency in U937, human PBMC and primary macrophages and immortalized murine monocytes. Since phosphorothioation of the end sequences of ORF4-PE did not impede its efficiency in stimulated U937 cells, this antisense molecule was used in all subsequent experiments. Phosphorothioation of ORF4 could potentially introduce non-specific effects (Hartmann et al., 1996), therefore, the specificity of ORF4-PE was further analyzed. ORF4-PE (5 μM), in the absence of

Lipofectin, was not toxic to U937 cells (data not shown) and displayed a dose response similar to that of ORF4 where 1 μM, 100 and 10 nM reduced TNF-α levels in stimulated U937 cells by 65 ± 5%, 36 ± 7% and 23 ± 8% respectively (Fig. 4a). To determine whether ORF4-PE influenced other inflammatory cytokine levels, IL-6 levels were measured in supernatants from stimulated U937 cells treated with ORF4-PE. ORF4-PE doses of 1 μM, 100 and 10 nM did not significantly affect IL-6 levels in the supernatants of stimulated U937 cells (Fig. 4a).

To determine the efficiency of ORF4-PE in primary human cells under different stimulatory conditions, primary cells were treated with ORF4-PE and stimulated with PMA/PHA (10 ng/5 μg/ml; PBMC) or with LPS (1 μg/ml; macrophages). ORF4-PE (1 μM) treatment of PBMC cultures significantly decreased PMA/PHA TNF-α gene expression by 62 ± 9% ($p<0.001$) (Fig 4b). ORF4-PE (1 μM) treatment of LPS-stimulated primary macrophages significantly decreased TNF-α levels by 73 ± 8 % ($p< 0.00004$)(Fig. 4b). In both PBMC and primary macrophages, 100 and 10 nM ORF4-PE showed a trend of dose-dependent reduction of TNF-α levels (Fig. 4b). All PBMC (n=5) and primary macrophage cultures (n=5), were susceptible to ORF4-PE treatment with inhibition of TNF-α ranging approximately from 50-70% in PBMC cultures and from 70-85% in primary

macrophages (data not shown). ORF4-PE efficiency of TNF-α reduction was approximately 10-15% greater in primary macrophage cultures compared to PBMC or U937 cells, possibly due to active AS-ODN/Lipofectin phagocytosis by primary macrophages (Chaudhuri, 1997; Iversen et al., 1992). Finally, since the TNF-α gene sequence is highly conserved across species, the relative cross-species efficiency of ORF4-PE was tested in murine monocytes treated with ORF4-PE and stimulated with LPS. ORF4-PE (1 μM) significantly reduced murine TNF-α levels in LPS-stimulated cells by 48 ± 8 % ($p<0.009$) (Fig. 4b). Thus, in both human and murine cells, exon sequences upstream of the donor splice site of the 2nd exon of TNF-α are highly susceptible to AS-ODN actions.

In summary, exon sequences upstream of splice sites play a critical role in mRNA processing. Correct mRNA processing is dependent on spliceosome interactions with these sites. Using antisense oligodeoxynucleotides (AS-ODNs), these and other sequences of the proinflammatory tumor necrosis factor alpha (TNF-α) gene were targeted because it is multiply spliced and has been difficult to regulate with AS-ODNs in the past. AS-ODNs targeting exon sequences upstream of the donor splice sites of internal exons 2 (ORF4) and 3 (ORF6) significantly reduced TNF-α levels in stimulated U937 cells by 62 ± 7%

and 51 ± 9%, respectively, in a dose-dependent manner but did not affect IL-6 levels (see Tables). In contrast, AS-ODNs targeting the exon sequences downstream of the acceptor splice sites of exon 1, 2 and 3 failed to reduce TNF-α levels significantly under the same conditions. End-phosphorothioated ORF4 (ORF4-PE) significantly reduced TNF-α mRNA levels by greater than 80% ($p<0.001$) and protein levels by 60% ($p<0.001$) in U937 cells. ORF4-PE reduced newly synthesized TNF-α protein levels by greater than 80% in LPS-stimulated human macrophages, by greater than 60% in PMA/PHA-stimulated human PBMC and by approximately 50% in LPS-stimulated murine monocytes. These results show that exon sequences flanking donor splice sites provide highly vulnerable target domains for antisense inhibition of TNF-α gene expression.

Throughout this application, various publications, including United States patents, are referenced either by number or by author and year and patents by number. Full citations for the publications are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology

which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

Table 1. ODN sequence, target domain and % inhibition of TNF-α.

AS-ODN	Sequence[1] / SEQ ID No:	Mer	TNF-α Position[2]	% inhibition[3] (mean ± SD)
ORF1	GCC AGC TCC ACG TCC CGG ATC ATG CTT TCA **SEQ ID No:1**	30	+9- +38	25 ± 5
ORF2	GGC TGA GGA ACA AGC ACC GCC TGG AG **SEQ ID No:2**	26	+78- +103	16 ± 6
ORF3	GAC TCT TCC CTC TGG GGG CCG ATC ACT CCA **SEQ ID No:3**	30	+159- +188	25 ± 5
ORF4	CTG ACT GCC TGG GCC AGA GGG CTG ATT AG **SEQ ID No:4**	29	+205- +233	62 ± 7**
ORF5	TCG GGG TTC GAG AAG ATG ATC TGA CTG C **SEQ ID No:5**	28	+226- +253	14 ± 4
ORF6	CCA CAT GGG CTA CAG GCT TGT CAC TCG **SEQ ID No:6**	27	+251- +277	51 ± 9*
ORF7	GCT TGA GGG TTT GCT ACA ACA TGG GCT ACA **SEQ ID No:7**	30	+264- +293	18 ± 6
ORF8	GGC CCG GCG GTT CAG CCA CTG GAG **SEQ ID No:8**	24	+304- +327	36 ± 4
ORF9	CAC GCC ATT GGC CAG GAG GGC ATT GG **SEQ ID No:9**	26	+326- +351	30 ± 3
ORF10	AGG TAC AGG CCC TCT GAT GGC ACC ACC AG **SEQ ID No:10**	29	+370- +398	25 ± 5
ORF11	CCT GTC TTC TTG GGG AGC GCC TCC TC **SEQ ID No:11**	26	+40- +65	22 ± 6
ORF14	CTG GGG CCC CCC TGT CTT CTT GGG GA **SEQ ID No:12**	26	+50- +75	15 ± 8
ORF15	GCC TGG AGC CCT GGG GCC CCC CTG TC **SEQ ID No:13**	26	+60- +85	19 ± 7
ORF16	ACA AAG CAC CGC CTG GAG CCC TGG GG **SEQ ID No:14**	26	+70- +95	24 ± 6
ORF17	GGA AGG AGA AGA GGC TGA GGA ACA **SEQ ID No:15**	24	+92- +115	22 ± 5
ORF18	TGC CAC GAT CAG GAA GGA GAA **SEQ ID No:16**	21	+106- +126	25 ± 8
ORF21	GCA GCA GGA AGA AGA GCG TGG TG **SEQ ID No:17**	23	+132- +154	28 ± 4
O-3'UTR	AAT AAT AAA TAA ATA ATA AAT AAT CAC AAG **SEQ ID No:18**	31	+1310- +1339	21 ± 5

ODN 5′A[5]	CAT GCT TTC AGT CAT **SEQ ID No:19**	15	5′ AUG start	26 ± 8
ODN 5′B[6]	TGT GCT CAT GGT GTC TTT **SEQ ID No:20**	18	5′ AUG start	27 ± 9
Controls				
ORF4MM1[4]	CTG ACA TCC TGG GCC **CC**A GGG CTG ATT AG **SEQ ID No:21**	29	+205- +233	22 ± 9
ORF4MM2[4]	CTG ACT GCC TG**C** TCC AGA GGG CTG ATT **SEQ ID No:22**	27	+207- +233	28 ± 8
O-8433	ATC GTC CGG ATC TGT CTC TGT **SEQ ID No:23**	21	HIV-1 Tat	19 ± 4

1. Antisense ODN sequences are shown in a 5' to 3' direction.
2. Positions are numbered relative to the 5' AUG sequence of TNF-α.
3. U937 cells were treated with 1 μM ODN and percent inhibition of TNF-α was corrected for the presence of Lipofectin.
4. Mismatches of bases within ORF4 are denoted in bold.
5. Hartmann et al. 1996.
6. Rojanasakul et al. 1997

** ($p \leq 0.001$).

Table 2. ODNs sequence, target site within the second or third exon and % inhibition of TNF-α.

AS-ODN	Sequence[1] / SEQ ID No:	Mer	TNF-α position[2]	Flanking[3] splice site	% inhibition[4] (mean ± SD)
			exon 2		
ORF4-PE	**CTG** ACT GCC TGG GCC AGA GGG CTG ATT **AG** **SEQ ID No:4**	29	+205- +233	donor	65 ± 5**
ORF4-PR	CTG ACT GCC **TGG** GCC AGA GGG CTG **ATT** AG **SEQ ID No:4**	29	+205- +233	donor	42 ± 5*
O4.5	GAT TAG AGA GAG GTC CCT GGG **SEQ ID No:24**	21	+190- +210	acceptor	32 ± 6
O4.10	TGG GCC AGA GGG CTG A **SEQ ID No:25**	16	+209- +224	mid exon	31 ± 7
O4.4	AGG GCT GAT TAG AGA GAG GTC **SEQ ID No:26**	21	+195- +216	mid exon	31 ± 8
O4.1	TGC CTG GGC CAG AGG GCT GAT TAG **SEQ ID No:27**	24	+205- +228	donor	43 ± 5*
O4.2	CTG ACT GCC TGG GCC AGA GGG CTG **SEQ ID No:28**	24	+210- +233	donor	42 ± 4*
O4.3	ACT GCC TGG GCC AGA GGG CTG **SEQ ID No:29**	21	+210- +230	donor	39 ± 5*
O4.7	TTC GAG AAG ATG ATC TGA CTG **SEQ ID No:30**	21	+227- +247	donor	44 ± 8*
			exon 3		
O4.6	GAA GAT GAT CTG ACT GCC TGG **SEQ ID No:31**	21	+222- +242	acceptor	28 ± 4
O4.8	GGG GTT CGA GAA GAT GAT **SEQ ID No:32**	18	+233- +251	acceptor	34 ± 5
O4.9	CTT GTC ACT CGG GGT TCG **SEQ ID No:33**	18	+244- +261	mid exon	32 ± 4

1. Antisense ODN sequences are shown in a 5' to 3' direction. Phosphorothioated nucleotides are denoted in bold font.
2 Positions are numbered relative to the 5' AUG sequence of TNF-α.
3 ODNs were designed to target exon sequences flanking donor or acceptor sites of the internal exons of TNF-α.
4 U937 cells were treated with 1 μM ODN and % inhibition of TNF-α was corrected for the presence of Lipofectin.
* ($p \leq 0.01$) ** ($p \leq 0.001$).

REFERENCES

Aggarwal, B. B., Schwarz, L., Hogan, M. E., and Rando, R. F. (1996). Triple helix-forming oligodeoxyribonucleotides targeted to the human tumor necrosis factor (TNF) gene inhibit TNF production and block the TNF-dependent growth of human glioblastoma tumor cells. Cancer Res *56*, 5156-5164.

Agrawal, 1996. Antisense oligonucleotides: towards clinical trials, TIBTECH, 14:376.

Arima, H., Aramaki, Y., and Tsuchiya, S. (1997). Effects of oligodeoxynucleotides on the physicochemical characteristics and cellular uptake of liposomes. J Pharm Sci *86*, 438-442.

Arvin, B., Neville, L. F., Barone, F. C., and Feuerstein, G. Z. (1996). The role of inflammation and cytokines in brain injury. Neurosci Biobehav Rev *20*, 445-452.

Bell, H., Kimber, W. L., Li, M., and Whittle, I. R. (1998). Liposomal transfection efficiency and toxicity on glioma cell lines: *in vitro* and *in vivo* studies. Neuroreport *9*, 793-798.

Beutler, B., and Grau, G. E. (1993). Tumor necrosis factor in the pathogenesis of infectious diseases. Crit Care Med *21*, S423-435.

Beutler, B., and van Huffel, C. (1994). Unraveling function in the TNF ligand and receptor families. Science *264*, 667-668.

Boeve, C. M., and De Ley, M. (1994). Inhibition of human interferon-gamma expression by antisense oligodeoxynucleotides. J Leukoc Biol *55*, 169-174.

Chaudhuri, G. (1997). Scavenger receptor-mediated delivery of antisense mini-exon phosphorothioate oligonucleotide to Leishmania-infected macrophages. Selective and efficient elimination of the parasite. Biochem Pharmacol *53*, 385-391.

Chen, P., Mayne, M., Power, C., and Nath, A. (1997). The Tat protein of HIV-1 induces tumor necrosis factor-a production: Implications for HIV-1 associated neurological diseases. J Biol Chem *272*, 22385-22388.

Dominski, Z., and Kole, R. (1996). Effects of exon sequences on splicing of model pre-mRNA substrates *in vitro*. Acta Biochim Pol *43*, 161-173.

Dominski, Z., and Kole, R. (1994). Identification and characterization by antisense oligonucleotides of exon and intron sequences required for splicing. Mol Cell Biol *14*, 7445-7454.

Dominski, Z., and Kole, R. (1994). Identification of exon sequences involved in splice site selection. J Biol Chem *269*, 23590-23596.

Dominski, Z., and Kole, R. (1991). Selection of splice sites in pre-mRNAs with short internal exons. Mol Cell Biol *11*, 6075-6083.

Fauci, A. S. (1996). Host factors and the pathogenesis of HIV-induced disease. Nature *384*, 529-534.

Ffrench-Constant, C. (1994). Pathogenesis of Multiple Sclerosis. Lancet *343*, 271-275.

Gao, W. Y., Han, F. S., Storm, C., Egan, W., and Cheng, Y. C. (1992). Phosphorothioate oligonucleotides are inhibitors of human DNA polymerases and RNase H: implications for antisense technology. Mol Pharmacol *41*, 223-229.

Gearing, A. J., Beckett, P., Christodoulou, M., Churchill, M., Clements, J. M., Crimmin, M., Davidson, A. H., Drummond, A. H., Galloway, W. A., Gilbert, R., and et al. (1995). Matrix metalloproteinases and processing of pro-TNF-alpha. J Leukoc Biol *57*, 774-777.

Givner, L. B., Gray, L., and TM, O. S. (1995). Antibodies to tumor necrosis factor-alpha: use as adjunctive therapy in established group B streptococcal disease in newborn rats. Pediatr Res *38*, 551-554.

Gough, N. (1988). Rapid and quantitative preparation of cytoplasmic RNA from small numbers of cells. Anal Biochem *173*, 93-95.

Hanecak, R., Brown Driver, V., Fox, M. C., Azad, R. F., Furusako, S., Nozaki, C., Ford, C., Sasmor, H., and Anderson, K. P. (1996). Antisense oligonucleotide inhibition of hepatitis C virus gene expression in transformed hepatocytes. J Virol *70*, 5203-5212.

Hartmann, G., Krug, A., Eigler, A., Moeller, J., Murphy, J., Albrecht, R., and Endres, S. (1996). Specific suppression of human tumor necrosis factor-alpha synthesis by antisense oligodeoxynucleotides. Antisense Nucleic Acid Drug Dev *6*, 291-299.

Hartmann, G., Krug, A., Waller Fontaine, K., and Endres, S. (1996). Oligodeoxynucleotides enhance lipopolysaccharide-stimulated synthesis of tumor necrosis factor: dependence on phosphorothioate modification and reversal by heparin. Mol Med *2*, 429-438.

Hawkins, J. D. (1988). A survey on intron and exon lengths. Nucleic Acids Res *16*, 9893-9908.

Hertel, K. J., Lynch, K. W., and Maniatis, T. (1997). Common themes in the function of transcription and splicing enhancers. Curr Opin Cell Biol *9*, 350-357.

Hodges, D., and Crooke, S. T. (1995). Inhibition of splicing of wild-type and mutated luciferase-adenovirus pre-mRNAs by antisense oligonucleotides. Mol Pharmacol *48*, 905-918.

Iversen, P. L., Zhu, S., Meyer, A., and Zon, G. (1992). Cellular uptake and subcellular distribution of phosphorothioate oligonucleotides into cultured cells. Antisense Res Dev *2*, 211-222.

Jacobson, A. B., and Zuker, M. (1993). Structural analysis by energy dot plot of a large mRNA. J Mol Biol *233*, 261-269.

Khaled, Z., Benimetskaya, L., Zeltser, R., Khan, T., Sharma, H. W., Narayanan, R., and Stein, C. A. (1996). Multiple mechanisms may contribute to the cellular anti-adhesive effects of phosphorothioate oligodeoxynucleotides. Nucleic Acids Res *24*, 737-745.

Khoury, G., Gruss, P., Dhar, R., and Lai, C. J. (1979). Processing and expression of early SV40 mRNA: a role for RNA conformation in splicing. Cell *18*, 85-92.

Krieg, A. M., Matson, S., Cheng, K., Fisher, E., Koretzky, G. A., and Koland, J. G. (1997). Identification of an oligodeoxynucleotide sequence motif that specifically inhibits phosphorylation by protein tyrosine kinases. Antisense Nucleic Acid Drug Dev 7, 115-123.

Krieg, A. M., Matson, S., and Fisher, E. (1996). Oligodeoxynucleotide modifications determine the magnitude of B cell stimulation by CpG motifs. Antisense Nucleic Acid Drug Dev *6*, 133-139.

Lappalainen, K., Miettinen, R., Kellokoski, J., Jaaskelainen, I., and Syrjanen, S. (1997). Intracellular distribution of oligonucleotides delivered by cationic liposomes: light and electron microscopic study. J Histochem Cytochem *45*, 265-274.

Laptev, A. V., Lu, Z., Colige, A., and Prockop, D. J. (1994). Specific inhibition of expression of a human collagen gene (COL1A1) with modified antisense oligonucleotides. The most effective target sites are clustered in double-stranded regions of the predicted secondary structure for the mRNA. Biochemistry *33*, 11033-11039.

Lefebvre d'Hellencourt, C., Diaw, L., Cornillet, P., and Guenounou, M. (1996). Inhibition of human TNF alpha and LT in cell-free extracts and in cell culture by antisense oligonucleotides. Biochim Biophys Acta *1317*, 168-174.

Liang, W. W., Shi, X., Deshpande, D., Malanga, C. J., and Rojanasakul, Y. (1996). Oligonucleotide targeting to alveolar macrophages by mannose receptor-mediated endocytosis. Biochim Biophys Acta *1279*, 227-234.

Lima, W. F., Monia, B. P., Ecker, D. J., and Freier, S. M. (1992). Implication of RNA structure on antisense oligonucleotide hybridization kinetics. Biochemistry *31*, 12055-12061.

Lupia, E., Montrucchio, G., Battaglia, E., Modena, V., and Camussi, G. (1996). Role of tumor necrosis factor-alpha and platelet-activating factor in neoangiogenesis induced by synovial fluids of patients with rheumatoid arthritis. Eur J Immunol *26*, 1690-1694.

Miraglia, L., Geiger, T., Bennett, F., and Dean, N. (1996). Inhibition of interleukin-1 type 1 receptor expression in human cell-lines by an antisense phosphorothioate oligodeoxynuclotide. Int. J. Immunopharmac. *18*, 227-240.

Mishra, R. K., Le Tinevez, R., and Toulme, J. J. (1996). Targeting nucleic acid secondary structures by antisense oligonucleotides designed through *in vitro* selection. Proc Natl Acad Sci U S A *93*, 10679-10684.

Mishra, R. K., and Toulme, J. J. (1994). *In vitro* selection of antisense oligonucleotides targeted to a hairpin structure. C R Acad Sci III *317*, 977-982.

Moreland, L. W., Baumgartner, S. W., Schiff, M. H., Tindall, E. A., Fleishmann, R. M., Weaver, A. L., Ettlinger, R. E., Cohen, S., Koopman, W. J., Mohler, K., Widmer, M. B., and Blosch, C. M. (1997). Treatment of Rheumatoid Arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein. N Engl J Med *337*, 141-147.

Moulds, C., Lewis, J. G., Froehler, B. C., Grant, D., Huang, T., Milligan, J. F., Matteucci, M. D., and Wagner, R. W. (1995). Site and mechanism of antisense inhibition by C-5 propyne oligonucleotides. Biochemistry *34*, 5044-5053.

Padgett, R. A., Grabowski, P. J., Konarska, M. M., Seiler, S., and Sharp, P. A. (1986). Splicing of messenger RNA precursors. Annu Rev Biochem *55*, 1119-1150.

Perez, J. R., Li, Y., Stein, C. A., Majumder, S., van Oorschot, A., and Narayanan, R. (1994). Sequence-independent induction of Sp1 transcription factor activity by phosphorothioate oligodeoxynucleotides. Proc Natl Acad Sci U S A *91*, 5957-5961.

Pfeffer, K., Matsuyama, T., Kundig, T. M., Wakeham, A., Kishihara, K., Shahinian, A., Wiegmann, K., Ohashi, P. S., Kronke, M., and Mak, T. W. (1993). Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell *73*, 457-467.

Power, C., McArthur, J. C., Johnson, R. T., Griffin, D. E., Glass, J. D., Dewey, R., and Chesebro, B. (1995). Distinct HIV-1 env sequences are associated with neurotropism and neurovirulence. Curr Top Microbiol Immunol *202*, 89-104.

Probert, L., Akassoglou, K., Kassiotis, G., Pasparakis, M., Alexopoulou, L., and Kollias, G. (1997). TNF-alpha transgenic and knockout models of CNS inflammation and degeneration. J Neuroimmunol *72*, 137-141.

Probert, L., Akassoglou, K., Pasparakis, M., Kontogeorgos, G., and Kollias, G. (1995). Spontaneous inflammatory demyelinating disease in transgenic mice showing central nervous system-specific expression of tumor necrosis factor alpha. Proc Natl Acad Sci U S A *92*, 11294-11298.

Rojanasakul, Y., Weissman, D. N., Shi, X., Castranova, V., Ma, J. K., and Liang, W. (1997). Antisense inhibition of silica-induced tumor necrosis factor in alveolar macrophages. J Biol Chem *272*, 3910-3914.

Selmaj, K., Raine, C. S., and Cross, A. H. (1991). Anti-tumor necrosis factor therapy abrogates autoimmune demyelination. Ann Neurol *30*, 694-700.

Sharief, M. K., and Hentges, R. (1991). Association between tumor necrosis factor-alpha and disease progression in patients with multiple sclerosis. N Engl J Med *325*, 467-472.

Staley, J. P., and Guthrie, C. (1998). Mechanical devices of the spliceosome: Motors, clocks, springs and things. Cell *92*, 315-326.

Stein, C. A., and Cheng, Y. C. (1993). Antisense oligonucleotides as therapeutic agents--is the bullet really magical? Science *261*, 1004-1012.

Talley, A. K., Dewhurst, S., Perry, S. W., Dollard, S. C., Gummuluru, S., Fine, S. M., New, D., Epstein, L. G., Gendelman, H. E., and Gelbard, H. A. (1995). Tumor necrosis factor alpha-induced apoptosis in human neuronal cells: protection by the antioxidant N-acetylcysteine and the genes bcl-2 and crmA. Mol-Cell-Biol *15*, 2359-2366.

Tarn, W. Y., and Steitz, J. A. (1997). Pre-mRNA splicing: the discovery of a new spliceosome doubles the challenge. Trends Biochem Sci *22*, 132-137.

Taylor, M. F., Paulauskis, J. D., Weller, D. D., and Kobzik, L. (1996). *In vitro* efficacy of morpholino-modified antisense oligomers directed against tumor necrosis factor-alpha mRNA. J Biol Chem *271*, 17445-17452.

Thierry, A. R., Rahman, A., and Dritschilo, A. (1993). Overcoming multidrug resistance in human tumor cells using free and liposomally encapsulated antisense oligodeoxynucleotides. Biochem Biophys Res Commun *190*, 952-960.

Tomioka, H., Maw, W. W., Sato, K., and Saito, H. (1996). The role of tumour necrosis factor-alpha in combination with interferon-gamma or interleukin-1 in the induction of immunosuppressive macrophages because of Mycobacterium avium complex infection. Immunology *88*, 61-67.

Tracey, K. J., and Cerami, A. (1994). Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. Annu Rev Med *45*, 491-503.

Uhlmann, E., Ryte, A., and Peyman, A. (1997). Studies on the mechanism of stabilization of partially phosphorothioated oligonucleotides against nucleolytic degradation. Antisense Nucleic Acid Drug Dev *7*, 345-350.

Wagner, R. (1994). Gene inhibition using antisense oligonucleotides. Nature *372*, 333-335.

Wang, A. M., Creasey, A. A., Ladner, M. B., Lin, L. S., Strickler, J., Van Arsdell, J. N., Yamamoto, R., and Mark, D. F. (1985). Molecular cloning of the complementary DNA for human tumor necrosis factor. Science *228*, 149-154.

Wesselingh, S. L., Power, C., Glass, J. D., Tyor, W. R., McArthur, J. C., Farber, J. M., Griffin, J. W., and Griffin, D. E. (1993). Intracerebral cytokine messenger RNA expression in acquired immunodeficiency syndrome dementia. Ann Neurol *33*, 576-582.

Yagi, K., Noda, H., Kurono, M., and Ohishi, N. (1993). Efficient gene transfer with less cytotoxicity by means of cationic multilamellar liposomes. Biochem Biophys Res Commun *196*, 1042-1048.

Yang, Y., Yelavarthi, K. K., Chen, H. L., Pace, J. L., Terranova, P. F., and Hunt, J. S. (1993). Molecular, biochemical, and functional characteristics of tumor necrosis factor-alpha produced by human placental cytotrophoblastic cells. J Immunol *150*, 5614-5624.

Zelphati, O., and Szoka, F. C., Jr. (1996). Intracellular distribution and mechanism of delivery of oligonucleotides mediated by cationic lipids. Pharm Res *13*, 1367-1372.

Zheng, Z. M., and Specter, S. (1996). Dynamic production of tumour necrosis factor alpha (TNF-alpha) messenger RNA, intracellular and extracellular TNF-alpha by murine macrophages and possible association with protein tyrosine phosphorylation of STAT1 alpha and ERK2 as an early signal. Immunology *87*, 544-550.

Akhter et al, 1991. Interactions of antisense DNA oligonucleotide analogs with phospholipid membranes (liposomes). Nuc. Res. 19:5551-5559.

Crooke, 1995. Progress in antisense therapeutics, Hematol. Pathol. 2:59.

Loke et al, 1989. Characterization of oligonucleotide transport into living cells. PNAS USA 86:3474.

Morrison, 1991. Suppression of basic fibroblast growth factor expression by antisense oligonucleotides inhibits the growth of transformed human astrocytes. J. Biol. Chem. 266:728.

Culver, 1998. Site-Directed recombination for repair of mutations in the human ADA gene. (Abstract) Antisense DNA & RNA based therapeutics, February, 1998, Coronado, CA.

CLAIMS

What is claimed is:

1. A Synthetic nuclease resistant antisense oligodeoxynucleotides having a nucleotide sequence selected from the group consisting of SEQ ID No:4 and SEQ ID No:6.

2. The synthetic nuclease resistant antisense oligodeoxynucleotides as set forth in claim 1 having phosphorothioate bonds linking between the four 3'-terminus nucleotide bases for providing nuclease resistance.

3. A pharmaceutical or medical composition comprising as active ingredient at least one synthetic nuclease resistant antisense oligodeoxynucleotide as set forth in claim 1 in a physiologically acceptable carrier or diluent.

4. The pharmaceutical composition as set forth in claim 1 comprising either SEQ ID No:4 or SEQ ID No:6 and at least one other non-control AS-ODN selected from Tables 1 and 2 wherein the percent inhibition is greater than 25%.

5. A synthetic nuclease resistant antisense oligodeoxynucleotide capable of selectively modulating human tumor necrosis factor alpha by targeting exon sequences flanking donor splice sites thereby regulating expression of TNF-α.

6. The synthetic nuclease resistant antisense oligodeoxynucleotides having a nucleotide sequence as set forth in claim 5 selected from the group consisting of SEQ ID No:4 and SEQ ID No:6.

7. A pharmaceutical composition for selectively ~~modulating~~ mammalian ~~tunor~~ necrosis factor alpha in a mammal in need of such treatment consisting of an effective amount of at least one active ingredient as set forth in claim 1 and a pharmaceutically physiologically acceptable carrier or diluent.

8. A pharmaceutical or medical composition comprising as active ingredient at least one synthetic nuclease resistant antisense oligodeoxynucleotides as set forth in claim 6 in a physiologically acceptable carrier or diluent.

9. A pharmaceutical composition for modulating human tumor necrosis factor alpha in a patient in need of such treatment consisting of

an effective amount of at least one active ingredient as set forth in claim 6 or a ribozyme comprising a sequence complementary to at least a portion of exon sequences flanking donor splice sites in TNF-α; and

a pharmaceutically physiologically acceptable carrier or diluent.

10. A method of modulating expression of human tumor necrosis factor alpha in a mammal by administering a pharmaceutical composition as set forth in claim 5.

11. A DNA expression sequence comprising a transcriptional initiation region and a sequence encoding an oligonucleotide as set forth in claim 5.

12. A vector comprising a DNA sequence according to claim 11.

ANTISENSE OLIGODEOXYNUCLEOTIDES REGULATING

EXPRESSION OF TNF-α

ABSTRACT OF THE DISCLOSURE

A synthetic nuclease resistant antisense oligodeoxynucleotide capable of selectively modulating expression of human tumor necrosis factor-alpha by targeting exon sequences flanking donor splice sites, thereby regulating expression of TNF-α in a patient in need of such therapy is provided. In an embodiment either AS-ODN having the sequence set forth in SEQ ID No:4 or SEQ ID No:6 or a combination thereof can be used. The AS-ODN is administered either as the active ingredient in a pharmaceutical composition or by utilizing gene therapy techniques as an expression vector.

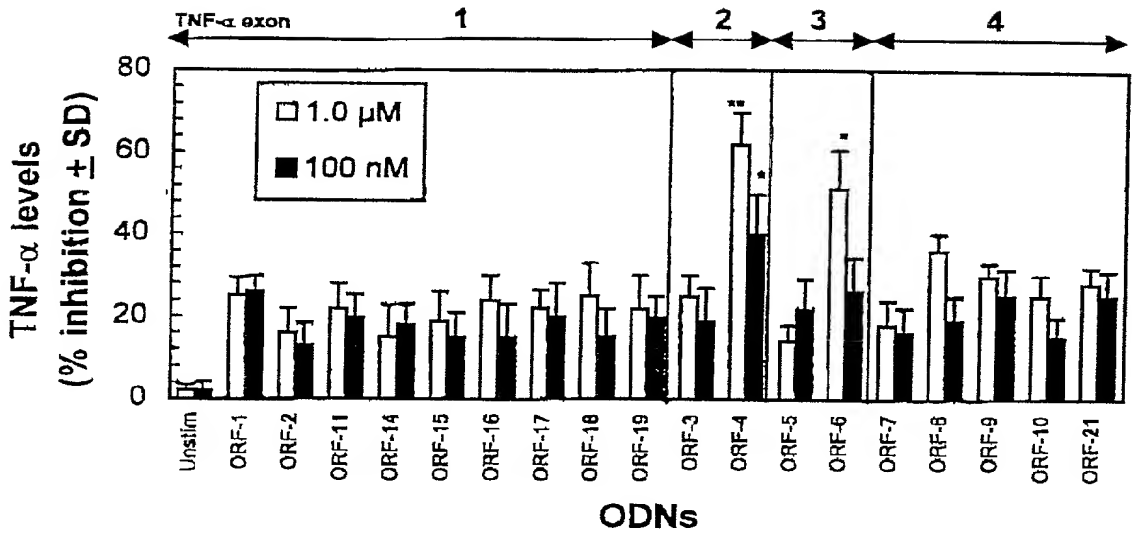

TNF-α exon
1
2
3
4
80
60
40
20
0
TNF-α levels
(% inhibition ± SD)
1.0 μM
100 nM
Unstim
ORF-1
ORF-2
ORF-11
ORF-14
ORF-15
ORF-16
ORF-17
ORF-18
ORF-19
ORF-3
ORF-4
ORF-5
ORF-6
ORF-7
ORF-8
ORF-9
ORF-10
ORF-21
ODNs

Fig-1

Fig-2A

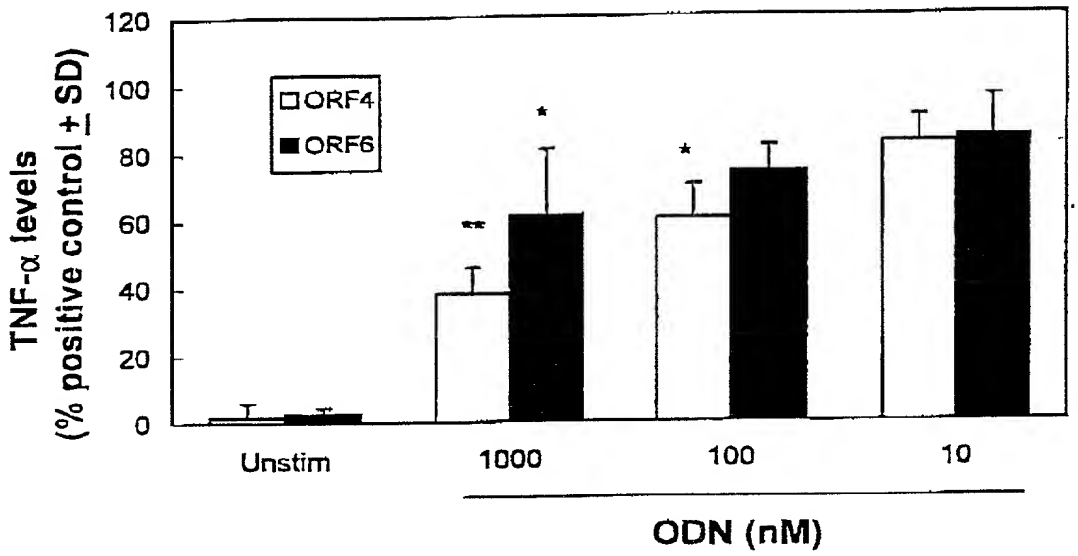
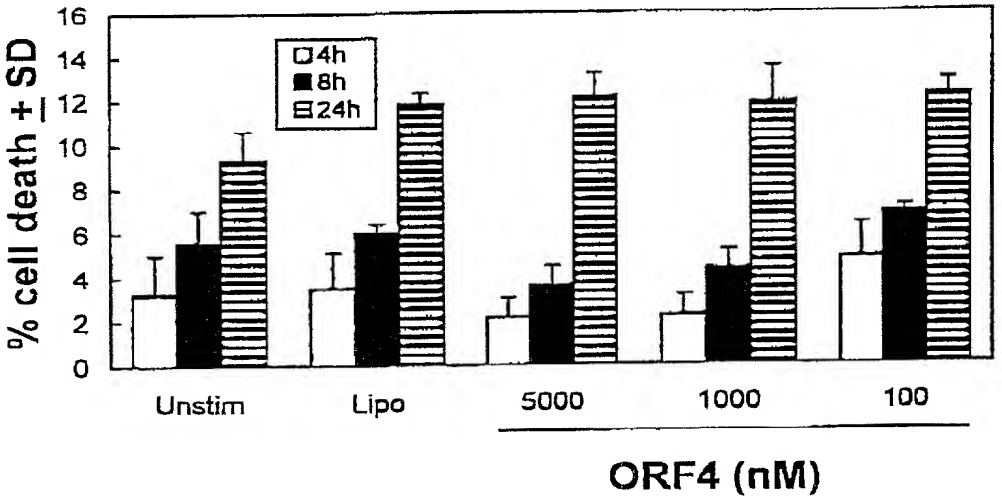

TNF-α levels
(% positive control ± SD)
120
100
80
60
40
20
0
ORF4
ORF6
*
**
*
Unstim
1000
100
10
ODN (nM)

Fig-2B


% cell death ± SD
16
14
12
10
8
6
4
2
0
4h
8h
24h
Unstim
Lipo
5000
1000
100
ORF4 (nM)

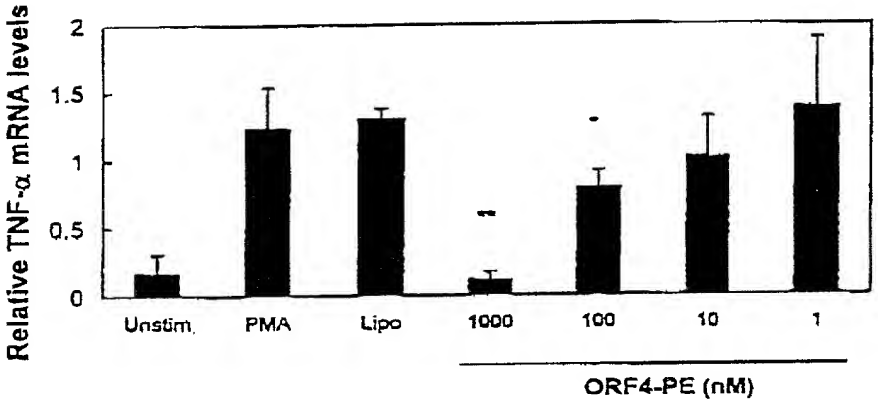

Relative TNF-α mRNA levels
2
1.5
1
0.5
0
Unstim.
PMA
Lipo
1000
100
10
1
ORF4-PE (nM)

Fig-3

Fig-4A

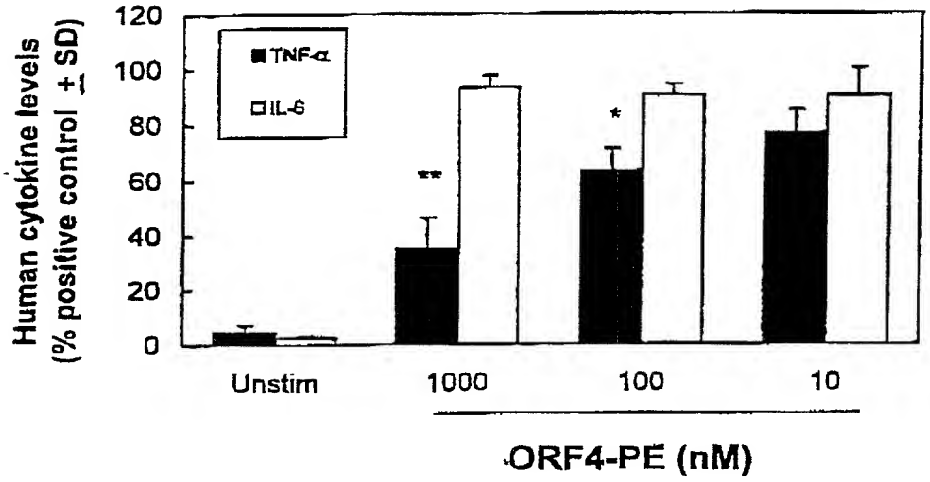
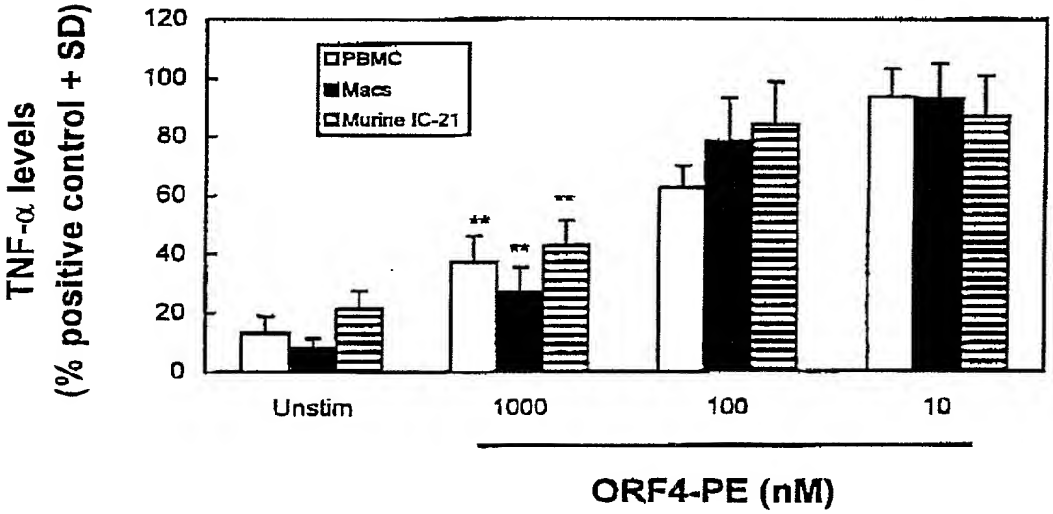

Human cytokine levels
(% positive control +1 SD)
120
100
80
60
40
20
0
TNF-α
IL-6
**
*
Unstim
1000
100
10
ORF4-PE (nM)

Fig-4B


TNF-α levels
(% positive control + SD)
120
100
80
60
40
20
0
PBMC
Macs
Murine IC-21
**
**
**
Unstim
1000
100
10
ORF4-PE (nM)

Docket No.
3045.00002

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Antisense Oligodeoxynucleotides Regulating Expression Of TNF-Alpha

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on October 22, 1998 as United States Application No. or PCT International Application Number 09/176,862

and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional

60/062,718	10-22-97
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

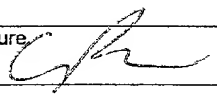
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

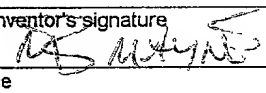
POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

Kenneth I. Kohn (30,955)
Ilene N. Montgomery (38,972)

Send Correspondence to: **Ilene N. Montgomery**
Kohn & Associates
30500 Northwestern Highway, Ste. 410
Farmington Hills, MI 48334

Direct Telephone Calls to: *(name and telephone number)*
Ilene N. Montgomery (248) 539-5050

Full name of sole or first inventor **Christopher Power**
Sole or first inventor's signature  Date 4 May 99
Residence **Calgary, Canada**
Citizenship **US and Canadian**
Post Office Address **134 Edgeview Road, NW**
Calgary, Canada T3A 4N1

Full name of second inventor, if any **Michael B. Mayne**
Second inventor's signature  Date 1/4/99
Residence **Winnipeg, Canada**
Citizenship **Canadian**
Post Office Address **589 Stradbrook Avenue**
Winnipeg, Canada R3L OK3